

# Screening for Estrogen and Androgen Receptor Activities in 200 Pesticides by *In Vitro* Reporter Gene Assays Using Chinese Hamster Ovary Cells

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We tested 200 pesticides, including some of their isomers and metabolites, for agonism and antagonism to two human estrogen receptor (hER) subtypes, hER $\alpha$  and hER $\beta$ , and a human androgen receptor (hAR) by highly sensitive transactivation assays using Chinese hamster ovary cells. The test compounds were classified into nine groups: organochlorines, diphenyl ethers, organophosphorus pesticides, pyrethroids, carbamates, acid amides, triazines, ureas, and others. These pesticides were tested at concentrations  $< 10^{-5}$  M. Of the 200 pesticides tested, 47 and 33 showed hER $\alpha$ - and hER $\beta$ -mediated estrogenic activities, respectively. Among them, 29 pesticides had both hER $\alpha$  and hER $\beta$  agonistic activities, and the effects of the organochlorine insecticides  $\beta$ -benzene hexachloride (BHC) and  $\delta$ -BHC and the carbamate insecticide methiocarb were predominantly hER $\beta$  rather than hER $\alpha$  agonistic. Weak antagonistic effects toward hER $\alpha$  and hER $\beta$  were shown in five and two pesticides, respectively. On the other hand, none of tested pesticides showed hAR-mediated androgenic activity, but 66 of 200 pesticides exhibited inhibitory activity against the transcriptional activity induced by 5 $\alpha$ -dihydrotestosterone. In particular, the antiandrogenic activities of two diphenyl ether herbicides, chlornitrofen and chlormethoxyfen, were higher than those of vinclozolin and *p,p'*-dichlorodiphenyl dichloroethylene, known AR antagonists. The results of our ER and AR assays show that 34 pesticides possessed both estrogenic and antiandrogenic activities, indicating pleiotropic effects on hER and hAR. We also discussed chemical structures related to these activities. Taken together, our findings suggest that a variety of pesticides have estrogenic and/or antiandrogenic potential via ER and/or AR, and that numerous other manmade chemicals may also possess such estrogenic and antiandrogenic activities. **Key words:** antiandrogenic activity, Chinese hamster ovary cells, estrogenic activity, human androgen receptor, human estrogen receptor  $\alpha$ , human estrogen receptor  $\beta$ , pesticide, reporter gene assay. *Environ Health Perspect* 112:524–531 (2004). doi:10.1289/ehp.6649 available via <http://dx.doi.org/> [Online 3 December 2003]

It has been well documented that several chemicals from agricultural, industrial, and household sources possess endocrine-disrupting properties, which provide a potential threat to human and wildlife reproduction (Colborn 1995; Colborn et al. 1993; Jensen et al. 1995). A suggested mechanism is that these environmental contaminants alter the normal functioning of the endocrine and reproductive system by mimicking or inhibiting endogenous hormone action, modulating the production of endogenous hormones, or altering hormone receptor populations (Sonnenschein and Soto 1998). A major mechanism of endocrine disruption is the action of chemicals as receptor agonists or antagonists through direct interaction with hormone receptors, thus altering endocrine function. In particular, chemicals mimicking endogenous estrogen via estrogen receptor (ER) have been the focus of research for the last 20 years. Meanwhile, recent studies have shown that several chemicals may exert antiandrogenic effect by interfering with androgen receptor (AR; Sohoni and Sumpter 1998; Vinggaard et al. 1999).

Pesticides commonly used to control agricultural and indoor pests are the most likely suspects as endocrine disruptors. The ubiquitous nature of pesticide usage with minimal precautions has resulted in contamination of

food, the workplace, and the environment. Recent reports showed that several pesticides exert estrogenic and antiandrogenic activities through interaction with estrogen and androgen receptors. To date, *p,p'*-dichlorodiphenyl trichloroethane (DDT) (Welch et al. 1969), methoxychlor (Bulger et al. 1978; Cummings 1997),  $\beta$ -benzene hexachloride (BHC) (Coosen and Velsen 1989), endosulfan, toxaphene, and dieldrin (Soto et al. 1995), and fenvalerate (Garey and Wolff 1998) have been reported as estrogenic pesticides. Recently, Andersen et al. (2002) have reported that several currently used pesticides, such as methiocarb, fenarimol, chlorpyrifos, deltamethrin, and tolclorfen-methyl, possess estrogenic activity on the basis of cell proliferation assay and transactivation assay using MCF-7 human breast cancer cells. On the other hand, studies have also revealed antiandrogenic pesticides, such as vinclozolin and *p,p'*-dichlorodiphenyl dichloroethylene (DDE) (Kelce et al. 1994, 1995), DDT isomer and methoxychlor (Maness et al. 1998), linuron (Gray et al. 1999; Lambright et al. 2000), procymidone (Ostby et al. 1999), and fenitrothion (Tamura et al. 2001). Andersen et al. (2002) reported that dieldrin, endosulfan, methiocarb, and fenarimol possessed antiandrogenic activity on the basis of transactivation assay using Chinese

hamster ovary (CHO) cells. Thus, estrogenic and antiandrogenic activities have been found in a number of pesticides, and it is conceivable that many other pesticides also have estrogenic and/or antiandrogenic activity.

Transactivation or reporter gene assay, which is a powerful tool for testing receptor agonists and antagonists among chemicals, has been established as a method for evaluating the receptor activity of chemicals. However, many compounds are independently evaluated for ER or AR activity by different reporter gene assays. This may lead to confusion in the evaluation of their potential as endocrine-disrupting chemicals. In addition, the recent cloning of a gene for a second estrogen receptor, ER $\beta$ , by Kuiper et al. (1996) led to the discovery that ER $\beta$  and the classic ER $\alpha$  differ in their ligand binding ability and transactivation properties (Kuiper et al. 1997; McInerney et al. 1998). Therefore, a screening system involving both ER subtypes ( $\alpha$  and  $\beta$ ) is required to completely evaluate the endocrine disruption potential of environmental estrogens. Previously, we reported highly sensitive reporter gene assays using CHO cells for detecting ER $\alpha$  and AR agonists/antagonists from chemicals, and demonstrated that the diphenyl ether-type herbicide chlornitrofen (CNP) and its amino derivatives (CNP-amino) possessed both antiandrogenic and estrogenic activities (Kojima et al. 2003). In the present study, we screened a total of 200 pesticides using our reporter gene assay systems for detecting two ER subtypes, ER $\alpha$  and ER $\beta$ , and AR activities. These pesticides, including several well-known estrogenic and antiandrogenic pesticides such as DDT and vinclozolin, were selected according to the frequency of their use in Japan and other countries, both currently and in the past. They are classified

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into nine groups according to similarities in their chemical structure and are discussed on the basis of the relationships between chemical structure and activity via hormone receptors. In this article, we provide evidence that a variety of pesticides have estrogenic and/or antiandrogenic potential via ER and/or AR and that their activities are related to chemical structure.

## Materials and Methods

**Chemicals.** 17 $\beta$ -Estradiol (E<sub>2</sub>; > 97% pure), 5 $\alpha$ -dihydrotestosterone (DHT; 95% pure), and tamoxifen citrate (98% pure) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The 200 pesticides tested in the present study are listed in Table 1. These pesticides were purchased from Wako, Sigma-Aldrich (St. Louis, MO, USA), Dr. Ehrenstorfer GmbH (Augsburg, Germany), AccuStandard Inc. (New Haven, CT, USA), and Hayashi Pure Chemical Industries, Ltd. (Osaka, Japan) and had a purity of 95–100%. Dimethylsulfoxide (DMSO) was also purchased as a vehicle from Wako.

E<sub>2</sub> and DHT were stored as a 1-mM stock solution in DMSO at –20°C. Pesticides were dissolved in DMSO to a final concentration of 10 mM, except for asulam, paraquat, diquat, iminocadine, glyphosate, and carben-dazim, which were directly dissolved in the medium, and all pesticides were diluted to the desired concentrations in phenol red-free Dulbecco's modified Eagle medium plus nutrient mixture Ham's F-12 (DMEM/F-12) immediately before use. The final solvent concentration in the culture medium did not exceed 0.1%, and this concentration did not affect cell yields.

**Cell line and cell culture conditions.** CHO-K1 cells were obtained from the Dainippon Pharmaceutical Co. (Osaka, Japan). Penicillin–streptomycin solution (antibiotics) and DMEM/F-12 were obtained from Gibco-BRL (Rockville, MD, USA). Fetal bovine serum (FBS) and charcoal-dextran-treated (CD) FBS were obtained from Hyclone (Logan, UT, USA). For routine maintenance, cells were grown in DMEM/F-12 supplemented with 10% FBS and antibiotics at 37°C in an atmosphere of 5% CO<sub>2</sub>/95% air under saturating humidity and passaged every week by trypsinization with 0.25% trypsin/0.02% ethylenediamine tetraacetic acid (EDTA) disodium salt solution (Life Technologies, Paisley, UK).

**Construction of plasmids.** The human ER $\alpha$  (hER $\alpha$ ) and AR (hAR) expression vectors (pcDNAER $\alpha$  and pZeoSV2AR) were constructed as previously described (Kojima et al. 2003). The hER $\beta$  expression vector was newly constructed as follows: The ER $\beta$  cDNA was cloned by reverse transcriptase–polymerase chain reaction from human placental RNA (Clontech, Palo Alto, CA, USA). The sequence

**Table 1.** The 200 pesticides tested in the reporter gene assays for hER $\alpha$ , hER $\beta$ , and hAR.

Group, compound	Group, compound	Group, compound
1. Organochlorines ( <i>n</i> = 29)	Isofenphos	Mepronil
Aldrin	Isoxathion	Metalaxyl
$\alpha$ -BHC	Leptophos	Metolachlor
$\beta$ -BHC	Malathion	Pretilachlor
$\gamma$ -BHC	Mecarbam	Propyzamide
$\delta$ -BHC	Methamidophos	Thenylchlor
Captan	Methodathion	7. Triazines ( <i>n</i> = 7)
<i>cis</i> -Chlordane	Methyl-parathion	Anilazine
<i>trans</i> -Chlordane	Monocrotophos	Atrazine
Chlorobenzilate	Parathion	Metribuzin
Chloropropylate	Phenthoate	Prometon
Chlorothalonil	Phorate	Prometryn
<i>o,p'</i> -DDT	Phosalone	Simazine
<i>p,p'</i> -DDT	Phosmet	Simetryn
<i>p,p'</i> -DDE	Piperophos	8. Ureas ( <i>n</i> = 8)
<i>p,p'</i> -DDD	Pirimiphos-methyl	Bensulfuron-methyl
Dichlobenil	Profenofos	Daimuron
Dicofol	Propaphos	Diflubenzuron
Dieldrin	Prothiofos	Diuron
$\alpha$ -Endosulfan	Prothiofos oxon	Linuron
$\beta$ -Endosulfan	Pyridaphenthion	Pencycuron
Endosulfan sulfate	Quinalphos	Prochloraz
Endrin	Terbufos	Propanil
Folpet	Tetrachlorvinphos	9. Others ( <i>n</i> = 44)
Fthalide	Thiometon	Amitraz
Heptachlor	Tolclofos-methyl	Benfuresate
Heptachlor epoxide	Tolclofos-methyl oxon	Bentazone
Methoxychlor	Trichlorfon	Benzoiximate
Pentachlorophenol	Vamidothion	Biphenyl
Quintozene	4. Pyrethroids ( <i>n</i> = 12)	Bitertanol
2. Diphenyl ethers ( <i>n</i> = 11)	Cyfluthrin	Bromopropylate
Acifluorfen	Cyhalothrin	Chinomethionat
Acifluorfen-methyl	Cypermethrin	Chloridazon
Bifenox	Deltamethrin	Dazomet
Chlormethoxyfen	Etofenprox	Diquat
Chlornitrofen	Fenvalerate	Ethoxyquin
Chlornitrofen-amino	Flucythrinate	Fenarimol
Chloroxuron	Fluvalinate	Ferimzone
Diclofop-methyl	Permethrin	Fluazinam
Fluazifop-butyl	Pyrethrin	Imazalil
Nitrofen	Tefluthrin	Imidacloprid
Oxyfluorfen	Tralomehrin	Iminocadine
3. Organophosphorus pesticides ( <i>n</i> = 56)	5. Carbamates ( <i>n</i> = 22)	Indanofan
Acephate	Bendiocarb	Ioxynil octanoate
Anilofos	Benomyl	Iprodione
Bromophos-ethyl	Carbaryl	Isoprothiolane
Bromophos-methyl	Carbendazim	Lenacil
Butamifos	Carbofuran	4-Chloro- <i>o</i> -toloxyacetic acid (MCPA)
Chlorpyrifos	Chlorpropham	2,4-Dichlorophenoxyacetic acid (2,4-D)
Chlorpyrifos-methyl	Diethofencarb	Paraquat
Cyanofenphos	Dimepiperate	Pendimethalin
Cyanophos	Esprocarb	2-Phenylphenol
Diazinon	Ethiofencarb	Probenazole
Dichlofenthion	Fenobucarb	Procymidone
Dichlorvos	Isoprocarb	Propiconazole
Dimethoate	Methiocarb	Pyrazolynate
Dioxabenzofos	Methomyl	Pyrazoxyfen
Disulfoton	Molinate	Pyroquilon
EPN	Oxamyl	Sethoxydim
Edifenphos	Phenmedipham	Thiabendazole
Ethion	Pirimicarb	Thiocyclam
Ethoprophos	Pyributicarb	Thiophanate-methyl
Fenamiphos	Thiobencarb	Triadimefon
Fenchlorphos	Thiobencarb sulfon	Triclazazole
Fenitrothion	Thiram	Triflumizole
Fenitrothion oxon	6. Acid amides ( <i>n</i> = 11)	Trifluralin
Fensulfotthion	Alachlor	Triforine
Fenthion	Asulam	Vinclozolin
Glyphosate	Cafenstrole	
Iprobenfos	Flutolanil	
	Mefenacet	

EPN, *O*-ethyl *O*-*p*-nitrophenyl phenylphosphonothioate.

of the cloned hER $\beta$  cDNA was verified and was inserted into the mammalian expression vector pcDNA3.1Zeo(-) (Invitrogen, San Diego, CA, USA), creating pcDNAER $\beta$ .

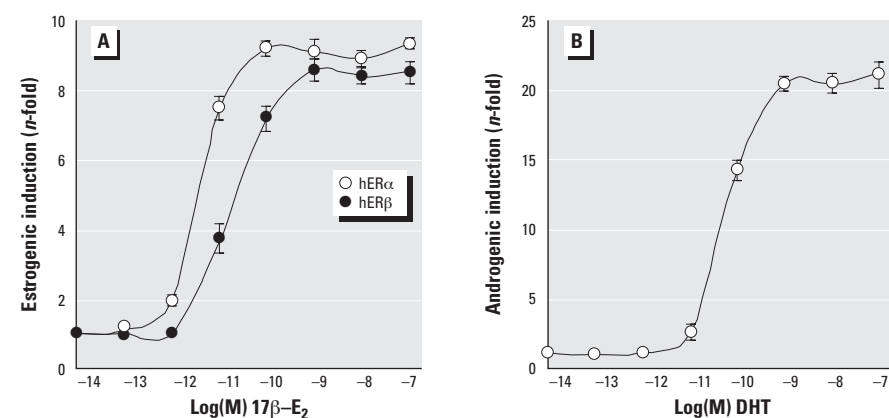
The estrogen-responsive element (ERE)-containing reporter plasmid pGL3-tkERE and the androgen-responsive element (ARE)-containing reporter plasmid pIND-ARE were constructed as described previously (Kojima et al. 2003). pRL-SV40 containing the *Renilla* luciferase gene was purchased from Promega (Madison, WI, USA) and used as an internal control for transfection efficiency.

**Reporter gene assays for hER $\alpha$ , hER $\beta$ , and hAR.** The host CHO-K1 cells were plated in 96-well microtiter plates (Nalge Nunc, Rochester, NY, USA) at a density of 8,400 cells/well in phenol red-free DMEM/F-12 containing 5% CD-FBS (complete medium) 1 day before transfection. For detection of hER $\alpha$  or hER $\beta$  activity, cells were transfected with 5 ng pcDNAER $\alpha$  or 5 ng pcDNAER $\beta$ , 50 ng pGL3-tkERE, and 5 ng pRL-SV40 per well using the transfection reagent FuGene6 (Roche Diagnostics Corp., Indianapolis, IN, USA). For detection of hAR activity, cells were transfected with 2.5 ng pZeoSV2AR, 50 ng pIND-ARE, and 5 ng pRL-SV40 per well. After a 3-hr transfection period, cells were dosed with various concentrations of test compounds or with 0.1% DMSO (vehicle control) in complete medium. For measurement of the antagonistic activity to hER $\alpha$ , hER $\beta$ , and hAR, either  $10^{-11}$  M E $_2$ ,  $10^{-10}$  M E $_2$ , or  $10^{-10}$  M DHT was added to the cell cultures along with the test compound, respectively (Figure 1). After an incubation period of 24 hr, cells were rinsed with phosphate-buffered saline (pH 7.4) and lysed with passive lysis buffer (50  $\mu$ L/well) provided with the Dual-Luciferase Reporter Assay kit (Promega). We measured the firefly luciferase activity with a MiniLumat LB 9506

luminometer (Berthold, Wildbad, Germany) before measuring *Renilla* luciferase activity in one reaction tube with 5- $\mu$ L aliquots of cell lysates using the Dual-Luciferase Reporter Assay kit, following the manufacturer's instructions. The firefly luciferase activity was normalized based on the *Renilla* luciferase activity of the cotransfected pRL-SV40. The values shown are mean  $\pm$  SD from at least three independent experiments.

We evaluated the results for the agonistic activities of the pesticides by relative activity, expressed as REC $_{20}$  (20% relative effective concentration)—that is, the concentration of the test compound showing 20% of the activity of  $10^{-10}$  M E $_2$ ,  $10^{-9}$  M E $_2$ , or  $10^{-9}$  M DHT for ER $\alpha$ , ER $\beta$ , or AR, respectively. When the activity of the test compound was higher than REC $_{20}$  within the concentration tested ( $\sim 10^{-8}$  to  $10^{-5}$  M), we judged the pesticide to be positive for activity. The results for the antagonistic activities of the pesticides were expressed as RIC $_{20}$  (20% relative inhibitory concentration), that is, the concentration of the test compound showing 20% inhibition of the activity induced by  $10^{-11}$  M E $_2$ ,  $10^{-10}$  M E $_2$ , or  $10^{-10}$  M DHT for ER $\alpha$ , ER $\beta$ , or AR, respectively. When the activity of the test compound was higher than the RIC $_{20}$  within the concentration tested, we judged the pesticide to be positive for inhibitory activity. To avoid cell toxicity by the pesticides, assays were performed for pesticides at concentrations  $\leq 10^{-5}$  M.

**Data analysis.** We evaluated the statistical significance of differences using the Student's *t*-test (two-tailed, equal variance) calculated by software (Excel; Microsoft, Redmond, WA, USA). The level of significance was  $p < 0.05$ . Data are presented as the mean and, where shown, the SD of at least three separate experiments with duplicate wells.



**Figure 1.** Dose-response curves of (A) 17 $\beta$ -E $_2$  and (B) 5 $\alpha$ -DHT in ER and AR assays. CHO cells were transiently transfected with expression plasmids for (A) hER $\alpha$  or hER $\beta$  or (B) hAR plus relative receptor-responsive firefly luciferase reporter plasmids and a constitutively active *Renilla* luciferase expression plasmid (transfection and toxicity control). Cells were treated with increasing concentrations of 17 $\beta$ -E $_2$  or 5 $\alpha$ -DHT to detect agonist activity. Firefly luciferase activity was normalized to *Renilla* luciferase activity. Values represent the mean  $\pm$  SD of three independent experiments and are presented as mean *n*-fold induction over the vehicle control.

## Results

**Response of 17 $\beta$ -E $_2$  in ER $\alpha$  and ER $\beta$  assays, and of 5 $\alpha$ -DHT in AR assay.** Figure 1A shows the dose-dependent transactivation of ER $\alpha$  and ER $\beta$  by 17 $\beta$ -E $_2$ , indicating that both receptors can be activated at very low hormone concentrations. The maximal ER $\alpha$  activity was achieved at  $10^{-10}$  M E $_2$  or more, exhibiting approximately 10-fold that of the control solvent. The maximal ER $\beta$  activity induced was 8.5-fold that of the solvent control at  $10^{-9}$  M E $_2$  or more. Thus, E $_2$  was more potent for ER $\alpha$  than for ER $\beta$ . From these dose-response curves, REC $_{20}$  values of E $_2$  for ER $\alpha$  and ER $\beta$  were deduced to be  $2.5 \times 10^{-12}$  M and  $5.3 \times 10^{-12}$  M, respectively.

Figure 1B shows the dose-dependent transactivation of AR by 5 $\alpha$ -DHT. Its activity was detectable from  $10^{-11}$  M DHT and reached a plateau at  $10^{-9}$  M DHT. The maximum induction was 21-fold that of the control solvent. The REC $_{20}$  value of DHT for AR was  $3.1 \times 10^{-11}$  M.

**Estrogenic effects of the pesticides.** Table 2 shows the REC $_{20}$  values and relative estrogenic activities at  $10^{-5}$  M of pesticides evaluated as positive for ER $\alpha$  agonistic activity. As shown in Table 2, 47 of the 200 pesticides were found to induce estrogenic activity in the ER $\alpha$  assay. A comparison of the potency of estrogenic activities among these active pesticides shows that the REC $_{20}$  values of *o,p'*-DDT,  $\beta$ -BHC, methoxychlor, and  $\alpha$ -endosulfan among the organochlorine pesticides, the CNP metabolite CNP-amino among the diphenyl ether pesticides, and butamifos among the organophosphorus pesticides were all lower than  $10^{-6}$  M, indicating that they possess potent estrogenic activity.

The results of ER $\beta$  agonistic activity are presented in Table 3. Thirty-three of 200 pesticides increased the ER $\beta$ -mediated transactivation gene response. Twenty-nine of these pesticides also have estrogenic activity via ER $\alpha$  (Table 2). The REC $_{20}$  values of  $\beta$ -BHC and *o,p'*-DDT among the organochlorine pesticides, CNP-amino among the diphenyl ether pesticides, and methiocarb among the carbamate pesticides were lower than  $10^{-6}$  M. However, butamifos, which showed potent ER $\alpha$  agonistic activity among the organophosphorus pesticides, was inactive in the ER $\beta$  assay. Dose-response curves of  $\beta$ -BHC,  $\delta$ -BHC, and methiocarb for ER $\alpha$  and ER $\beta$  are shown in Figure 2. These pesticides stimulated ER $\beta$  more strongly than they did ER $\alpha$ .

**Antiestrogenic effects of the pesticides.** Of 200 test pesticides, five (cyhalothrin, deltamethrin, alachlor, pyrazoxyfen, and triflumezole) showed antiestrogenic properties in the hER $\alpha$  transactivation assay with  $10^{-10}$  M E $_2$ . As shown in Figure 3A,  $10^{-5}$  M of these pesticides significantly inhibited the estrogenic response by  $10^{-11}$  M E $_2$ , as did the well-known

ER antagonist tamoxifen ( $10^{-8}$  and  $10^{-7}$  M). The RIC<sub>20</sub> values of cyhalothrin, deltamethrin, alachlor, pyrazoxyfen, triflumizole, and tamoxifen for hER $\alpha$  were  $9.0 \times 10^{-6}$  M,  $8.1 \times 10^{-6}$  M,  $4.5 \times 10^{-6}$  M,  $6.0 \times 10^{-6}$  M,  $9.8 \times 10^{-6}$  M, and  $3.2 \times 10^{-9}$  M, respectively.

In the hER $\beta$  transactivation assay of the 200 tested pesticides, only methoxychlor and pyrazoxyfen were antiestrogenic. As shown in Figure 3B,  $10^{-5}$  M methoxychlor or pyrazoxyfen inhibited by more than 20% the estrogenic activity induced by  $10^{-10}$  M E<sub>2</sub>. In this assay, tamoxifen also showed antiestrogenic activities at concentrations of  $10^{-8}$  and  $10^{-7}$  M. The RIC<sub>20</sub> values of methoxychlor, pyrazoxyfen, and tamoxifen for hER $\beta$  were  $9.0 \times 10^{-6}$  M,  $7.8 \times 10^{-6}$  M, and  $6.0 \times 10^{-9}$  M, respectively.

**Androgenic effects of the pesticides.** None of the pesticides tested showed androgenic transcriptional activity of more than 20% that induced by  $10^{-9}$  M DHT at the tested concentrations (data not shown).

**Antiandrogenic effects of the pesticides.** We tested 200 pesticides for their inhibitory effect on the androgenic activity induced by DHT ( $10^{-10}$  M). The RIC<sub>20</sub> values and relative luciferase activities (RLA) of 66 pesticides evaluated as having an inhibitory effect are summarized in Table 4. In particular, 13 pesticides (*o,p'*-DDT, *p,p'*-DDE, *p,p'*-DDT, chloropropylate, CNP, chlormethoxyfen, nitrofen, CNP-amino, oxyfluorfen, fenitrothion, vinclozolin, procymidone, and bromopropylate) showed a potent antiandrogenic effect with RIC<sub>20</sub> <  $10^{-6}$  M. Among these active pesticides, the RIC<sub>20</sub> of two diphenyl ether-type herbicides, CNP and chlormethoxyfen, were  $4.3 \times 10^{-8}$  M and  $6.8 \times 10^{-8}$  M, respectively, which is distinctly more potent than known AR antagonists such as vinclozolin and *p,p'*-DDE ( $1.6 \times 10^{-7}$  M and  $6.5 \times 10^{-7}$  M, respectively). In addition, weak antiandrogen effects with RIC<sub>20</sub> >  $10^{-6}$  M were found in 53 pesticides: 10 organochlorines, 2 diphenyl ethers, 18 organophosphorus pesticides, 4 pyrethroids, 2 carbamates, 3 acid amides, 5 ureas, and 9 others.

**Both ER agonists and AR antagonists in the pesticides.** Table 5 summarizes the 34 pesticides exhibiting dual activities as ER agonists and AR antagonists. Among these pesticides, organochlorine and organophosphorus pesticides were predominant. Above all, *o,p'*-DDT and CNP-amino were the most potent pesticides, having both estrogenic and antiandrogenic activities.

Cytotoxicity was not observed for any of the tested compounds at the selected dose range (data not shown).

## Discussion

To our knowledge, there are two reports on the screening of the endocrine-disrupting

effects of a large number of chemicals: on estrogenic activity of 514 chemicals using a yeast two-hybrid assay (Nishihara et al. 2000) and on binding ability of 188 chemicals to rat ER using a competitive binding assay (Blair et al. 2000). However, in these studies 118 pesticides of 514 chemicals and 20 pesticides of 188 chemicals showed little estrogenic activity or little binding ability to ER, respectively. In addition, there is no report on screening of ER $\beta$ -mediated estrogenic activity from a

**Table 2.** Responses induced by pesticide testing positive in the ER $\alpha$  transactivation assay.

Group <sup>a</sup> , compound	REC <sub>20</sub> <sup>b</sup> (M)	RLA <sup>c</sup> (%)
17 $\beta$ -E <sub>2</sub>	$2.5 \times 10^{-12}$	100 <sup>d</sup>
1. <i>o,p'</i> -DDT	$4.5 \times 10^{-8}$	93
$\beta$ -BHC	$3.5 \times 10^{-7}$	62
Methoxychlor	$5.6 \times 10^{-7}$	99
$\alpha$ -Endosulfan	$7.4 \times 10^{-7}$	91
<i>cis</i> -Chlordane	$1.1 \times 10^{-6}$	89
<i>p,p'</i> -DDT	$1.2 \times 10^{-6}$	83
<i>trans</i> -Chlordane	$1.3 \times 10^{-6}$	85
Dicofol	$1.8 \times 10^{-6}$	93
Endrin	$1.8 \times 10^{-6}$	55
Dieldrin	$2.0 \times 10^{-6}$	81
<i>p,p'</i> -DDE	$2.1 \times 10^{-6}$	62
Endosulfan sulfate	$2.2 \times 10^{-6}$	84
<i>p,p'</i> -DDD	$3.2 \times 10^{-6}$	55
Chloropropylate	$4.0 \times 10^{-6}$	63
$\delta$ -BHC	$6.0 \times 10^{-6}$	33
Heptachlor epoxide	$7.1 \times 10^{-6}$	31
Aldrin	$7.6 \times 10^{-6}$	28
Chlorobenzilate	$8.4 \times 10^{-6}$	24
2. CNP-amino	$3.7 \times 10^{-7}$	93
Chlornitrofen	$4.2 \times 10^{-6}$	50
Fluazifop-butyl	$7.8 \times 10^{-6}$	26
3. Butamifos	$6.7 \times 10^{-7}$	56
Prothiofos	$1.3 \times 10^{-6}$	120
Leptophos	$1.3 \times 10^{-6}$	96
Cyanofenphos	$2.1 \times 10^{-6}$	119
Ethion	$2.2 \times 10^{-6}$	74
Tolclofos-methyl	$2.5 \times 10^{-6}$	62
Bromophos-ethyl	$2.7 \times 10^{-6}$	65
EPN	$2.7 \times 10^{-6}$	99
Dichlofenthion	$3.7 \times 10^{-6}$	50
Quinalphos	$4.1 \times 10^{-6}$	78
Isoxathion	$4.2 \times 10^{-6}$	79
Pirimiphos-methyl	$4.8 \times 10^{-6}$	54
Bromophos-methyl	$5.7 \times 10^{-6}$	44
Isofenphos	$6.2 \times 10^{-6}$	38
Phenthoate	$6.6 \times 10^{-6}$	32
Chlorpyrifos	$7.5 \times 10^{-6}$	27
4. Fenvalerate	$3.7 \times 10^{-6}$	50
Flucythrinate	$5.7 \times 10^{-6}$	31
Cyfluthrin	$5.9 \times 10^{-6}$	45
Cypermethrin	$8.1 \times 10^{-6}$	28
Permethrin	$8.4 \times 10^{-6}$	24
5. Methiocarb	$7.2 \times 10^{-6}$	26
6. Thienylchlor	$1.6 \times 10^{-6}$	69
9. Pendimethalin	$1.7 \times 10^{-6}$	80
Bromopropylate	$2.5 \times 10^{-6}$	70
Fenarimol	$3.1 \times 10^{-6}$	73

Abbreviations: EPN, *O*-ethyl *O*-*p*-nitrophenyl phenylphosphonothioate; RLA, relative luciferase activity.

<sup>a</sup>Nine compound groups are listed in Table 1. <sup>b</sup>Concentration of the test compound showing 20% of the agonistic activity of  $10^{-10}$  M E<sub>2</sub>. <sup>c</sup>Percentage response at a concentration of  $10^{-5}$  M with 100% activity defined as the activity achieved with  $10^{-10}$  M E<sub>2</sub>. <sup>d</sup>RLA of E<sub>2</sub> is represented as the activity at a concentration of  $10^{-10}$  M.

large number of chemicals. We previously developed highly sensitive and specific reporter gene assays for ER $\alpha$  and AR (Kojima et al. 2003), and in the present study we established the ER $\beta$  assay by constructing the hER $\beta$  expression plasmid pcDNAER $\beta$  in our screening of 200 pesticides for their estrogenicity via hER $\alpha$ / $\beta$  and androgenicity via hAR. As a result, we found estrogenic activity for hER $\alpha$  in 47 pesticides and for hER $\beta$  in 33 pesticides, and antiestrogenic activity for hER $\alpha$  and hER $\beta$  in five and two pesticides, respectively. In the AR assay, although none of the tested pesticides showed AR agonistic activity, 66 of the 200 test pesticides surprisingly showed antiandrogenic activities. Thus, a number of pesticides were newly found to possess ER agonistic and/or AR antagonistic activities in addition to the pesticides already reported to be estrogenic and antiandrogenic. This suggests that our reporter gene assays are highly sensitive and specific.

We classified 200 pesticides into nine groups according to their chemical structure:

**Table 3.** Responses induced by pesticide testing positive in the ER $\beta$  transactivation assay.

Group <sup>a</sup> , compound	REC <sub>20</sub> <sup>b</sup> (M)	RLA <sup>c</sup> (%)
17 $\beta$ -E <sub>2</sub>	$5.3 \times 10^{-12}$	100 <sup>d</sup>
1. $\beta$ -BHC	$1.1 \times 10^{-7}$	122
<i>o,p'</i> -DDT	$1.2 \times 10^{-7}$	114
$\delta$ -BHC	$1.1 \times 10^{-6}$	164
<i>p,p'</i> -DDT	$1.7 \times 10^{-6}$	54
Dicofol	$1.9 \times 10^{-6}$	71
<i>p,p'</i> -DDD	$2.4 \times 10^{-6}$	65
<i>trans</i> -Chlordane	$3.1 \times 10^{-6}$	43
Endosulfan sulfate	$4.8 \times 10^{-6}$	54
$\gamma$ -BHC	$5.9 \times 10^{-6}$	43
Heptachlor epoxide	$6.3 \times 10^{-6}$	33
$\beta$ -Endosulfan	$6.2 \times 10^{-6}$	32
Heptachlor	$7.7 \times 10^{-6}$	29
<i>cis</i> -Chlordane	$4.9 \times 10^{-6}$	27
$\alpha$ -BHC	$8.3 \times 10^{-6}$	26
$\alpha$ -Endosulfan	$5.9 \times 10^{-6}$	26
Chloropropylate	$9.4 \times 10^{-6}$	22
<i>p,p'</i> -DDE	$1.0 \times 10^{-5}$	20
2. CNP-amino	$9.5 \times 10^{-7}$	121
3. Prothiofos	$1.7 \times 10^{-6}$	66
Bromophos-methyl	$3.1 \times 10^{-6}$	83
Tolclofos-methyl	$4.0 \times 10^{-6}$	73
Quinalphos	$6.7 \times 10^{-6}$	34
Leptophos	$6.8 \times 10^{-6}$	27
Cyanofenphos	$7.4 \times 10^{-6}$	27
Dichlofenthion	$8.3 \times 10^{-6}$	25
EPN	$8.7 \times 10^{-6}$	23
Ethion	$9.1 \times 10^{-6}$	22
Bromophos-ethyl	$9.7 \times 10^{-6}$	21
5. Methiocarb	$8.4 \times 10^{-7}$	106
6. Thienylchlor	$1.9 \times 10^{-6}$	47
9. Pendimethalin	$2.0 \times 10^{-6}$	75
Fenarimol	$4.1 \times 10^{-6}$	83
Bromopropylate	$8.2 \times 10^{-6}$	23

Abbreviations: EPN, *O*-ethyl *O*-*p*-nitrophenyl phenylphosphonothioate; RLA, relative luciferase activity.

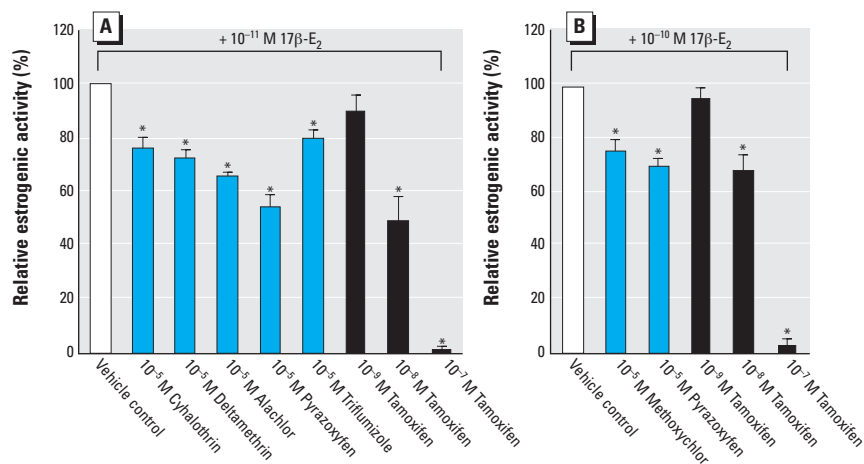
<sup>a</sup>Nine compound groups are listed in Table 1. <sup>b</sup>Concentration of the test compound showing 20% of the agonistic activity of  $10^{-9}$  M E<sub>2</sub>. <sup>c</sup>Percentage response at a concentration of  $10^{-5}$  M with 100% activity defined as the activity achieved with  $10^{-9}$  M E<sub>2</sub>. <sup>d</sup>RLA of E<sub>2</sub> is represented as the activity at a concentration of  $10^{-9}$  M.

organochlorines, diphenyl ethers, organophosphorus pesticides, pyrethroids, carbamates, acid amides, triazines, ureas, and others. Organochlorine-type pesticides should be of the most concern among the nine groups of pesticides suggested as candidates to be endocrine disruptors, because of their global distribution by widespread use and bioaccumulation through the ecosystem by high lipophilic property (Kutz et al. 1991; Simonich and Hites 1995). Several of these compounds (DDT, methoxychlor, BHC, endosulfan, and dieldrin) have been reported to possess estrogenic activity by studies with animals and cells (Bulger et al. 1978; Coosen and Velsen 1989; Soro et al. 1995; Welch et al. 1969). We have also demonstrated that *o,p'*-DDT,  $\beta$ -BHC, methoxychlor,  $\alpha$ -endosulfan, and *cis(trans)*-chlordane exert transcriptional reporter activity via ER $\alpha$  and/or ER $\beta$  by our assays. In addition, we newly identified estrogenic organochlorine pesticides such as dicofol, chloropropylate, and chlorobenzilate, whose chemical structures resemble those of DDT and its isomer (Figure 4). Among BHC isomers,  $\beta$ -BHC is most prevalent in the fatty tissues because of its greater stability, lipophilicity, and accumulation potential (Dejonckheere et al. 1978). In the present study,  $\beta$ -BHC and  $\delta$ -BHC exerted potent estrogenic activity especially via ER $\beta$  (Figure 2) but showed no effect in the androgen assay. Moreover, we also found 14 antiandrogenic pesticides among the organochlorine pesticides. The antiandrogenic properties of DDT isomers, methoxychlor, dieldrin, and endosulfan have already been reported (Andersen et al. 2002; Kelce et al. 1995; Maness et al. 1998), but in the present study several other pesticides were newly defined as AR antagonists. Although many organochlorine pesticides have weak hormonal activity, their lipophilic nature and long half-lives allow them to accumulate in

the fatty tissues of the body, increasing their concentration and bioavailability.

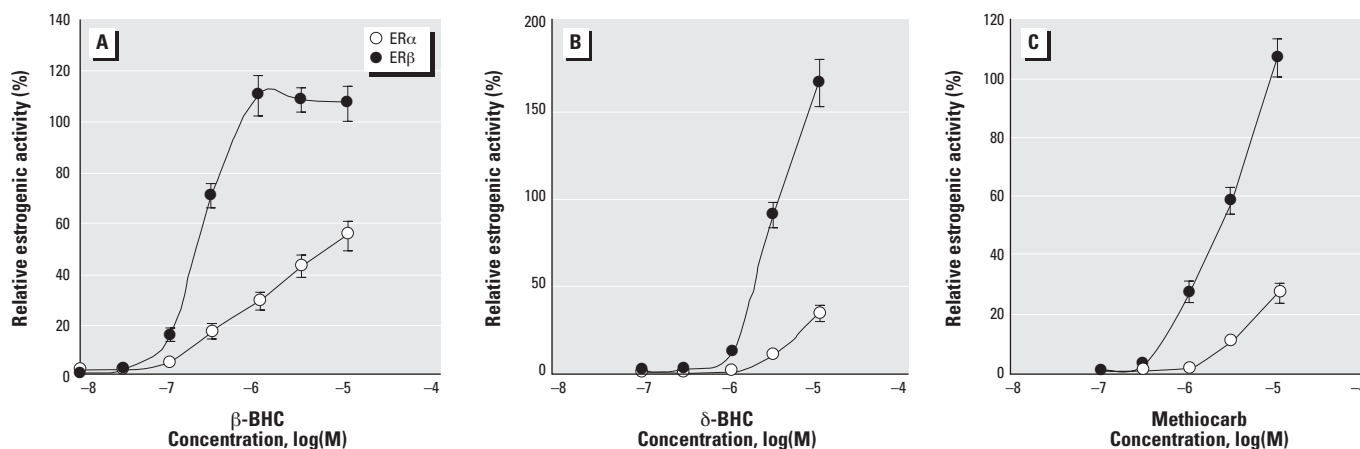
Diphenyl ether pesticides are no longer used, but a few decades ago this type of chemical was extensively used as an herbicide due to its low cost and toxicity. We have reported that a diphenyl ether-type pesticide, CNP, and its amino derivative (CNP-amino) act as both ER $\alpha$  agonists and AR antagonists *in vitro* (Kojima et al. 2003). In the present study, we additionally found that CNP-amino, but not CNP, possessed an ER $\beta$  agonistic effect, and that except for fluzifop-butyl, which demonstrated the weak ER $\alpha$  agonistic activity, no other diphenyl ether-type pesticides

showed estrogenic activity. This suggests that CNP-amino itself, rather than the structure of diphenyl ether, may contribute to the transactivation of ERs. Nevertheless, the greatest concern of diphenyl ether pesticides as endocrine-disrupting agents should reside in their potent AR antagonist activity. Recently, Tomura et al. (2001) demonstrated that a diphenyl ether herbicide, nitrofen, was antiandrogenic by transactivation assay and three-dimensional image analysis using COS-7 simian renal carcinoma cells. In the present study, seven of 11 diphenyl ether pesticides, including CNP, CNP-amino, and nitrofen showed antiandrogenic activity, and in



**Figure 3.** Antagonistic effects of pesticides in the hER $\alpha$  and hER $\beta$  transactivation assays. (A) CHO cells, transiently cotransfected with pcDNAER $\alpha$ , pGL3-tkERE, and pRL-SV40, were incubated with the vehicle control (0.1% DMSO) or 10<sup>-5</sup> M of cyhalothrin, deltamethrin, alachlor, pyrazoxyfen, or trifluzimole in the presence of 10<sup>-11</sup> M E<sub>2</sub>. Effect of ER antagonist tamoxifen (~10<sup>-9</sup> to 10<sup>-7</sup> M) was also measured as a positive control. Values represent the mean  $\pm$  SD of three independent experiments and are presented as percent induction, with 100% activity defined as the activity achieved with 10<sup>-11</sup> M E<sub>2</sub>. (B) CHO cells, transiently cotransfected with pcDNAER $\beta$ , pGL3-tkERE, and pRL-SV40, were incubated with the vehicle control (0.1% DMSO) or 10<sup>-5</sup> M of methoxychlor or pyrazoxyfen in the presence of 10<sup>-10</sup> M E<sub>2</sub>. Effect of ER antagonist tamoxifen (~10<sup>-9</sup> to 10<sup>-7</sup> M) was also measured as a positive control. Values represent the mean  $\pm$  SD of three independent experiments and are presented as percent induction, with 100% activity defined as the activity achieved with 10<sup>-10</sup> M E<sub>2</sub>.

\*Significantly different ( $p < 0.05$ ) from vehicle control (=100%).



**Figure 2.** Dose–response curves of (A)  $\beta$ -BHC, (B)  $\delta$ -BHC, and (C) methiocarb on hER $\alpha$ - and hER $\beta$ -mediated estrogenic activities. CHO cells were transiently transfected with pcDNAER $\alpha$  or pcDNAER $\beta$ , pGL3-tkERE, and pRL-SV40 as described in “Materials and Methods.” Cells were incubated with various concentrations of (A)  $\beta$ -BHC, (B)  $\delta$ -BHC, or (C) methiocarb. Values represent the mean  $\pm$  SD of three independent experiments and are presented as percent induction, with 100% activity defined as the activity achieved with 10<sup>-10</sup> M and 10<sup>-9</sup> M E<sub>2</sub> for ER $\alpha$  and ER $\beta$ , respectively.

particular, the antiandrogenic activities of CNP and chlomethoxyfen were more potent than those of known AR antagonists such as vinclozolin and *p,p'*-DDE. Chlomethoxyfen is structurally similar to CNP and nitrofen as well as the antiandrogen drug flutamide and the organophosphorus insecticide fenitrothion, all of which commonly contain nitrobenzene in the molecular structure (Figure 4). This may be the key point in screening AR antagonists from the multitude of chemicals (Kojima et al. 2003).

Organophosphorus pesticides are widely used in both agriculture and pest control. We found that a number of organophosphorus-type pesticides possess estrogenic and/or antiandrogenic activities. To date, it has been reported that tolclofos-methyl and quinalphos act as ER agonists (Andersen et al. 2002; Chatterjee et al. 1992) and that fenitrothion, parathion, and methyl parathion act as AR antagonists (Sohoni et al. 2001; Tamura et al. 2001). We also found that tolclofos-methyl and quinalphos have estrogenic activities via ER $\alpha$  and ER $\beta$ , and that fenitrothion, parathion, and methyl parathion have antiandrogenic activities via AR. Moreover, here we provide

new evidence that butamifos, prothiofos, leptophos, cyanofenphos, ethion, bromophosethyl, *O*-ethyl *O*-*p*-nitrophenyl phenylphosphonothioate (EPN), and dichlofenthion induce ER $\alpha$ -mediated transcriptional activity at concentrations lower than that of quinalphos. In addition, we found that 19 organophosphorus pesticides, including fenitrothion, parathion, and methyl parathion, possess antiandrogenic activity. Thus, the similarity in chemical structure among these pesticides may be the primary cause for their estrogenicity and/or antiandrogenicity. Interestingly, organophosphorus pesticides displaying these effects commonly contain a thiophosphoryl residue (P = S), as shown in Figure 4, whereas pesticides having an oxophosphoryl residue (P = O) such as prothiofos oxon, tolclofos-methyl oxon, acephate, and propaphos show few effects. This indicates that the estrogenic and/or antiandrogenic activities of parent compounds may disappear through oxidizing metabolism in the environment or body.

Pyrethroid and organophosphorus pesticides are the pesticides used most in Japan and other countries. Several pyrethroid pesticides (fenvalerate, permethrin, and cypermethrin)

have been reported to show estrogenic activities in MCF-7 cell proliferation and transactivation assays (Chen et al. 2002; Garey and Wolff 1998; Go et al. 1999). In contrast, Saito et al. (2000) reported that fenvalerate did not have estrogenic activity *in vitro* at a concentration of  $10^{-5}$  M. In our assays, five pyrethroid pesticides including fenvalerate were shown to increase transcriptional activity via ER $\alpha$ , and two pyrethroid pesticides decreased ER $\alpha$  activity induced by  $10^{-11}$  M E<sub>2</sub>. This suggests that several pyrethroid pesticides may act as weak ER agonists or antagonists. In addition, four pyrethroid pesticides were newly found to have weak antiandrogenic activity, and three of them (fenvalerate, flucythrinate, and cyfluthrin; Figure 4) displayed pleiotropic effects via both ER $\alpha$  and AR.

Klotz et al. (1997) reported that several carbamate insecticides (e.g., carbaryl, methomyl,

**Table 4.** Inhibitory effects of 66 pesticides on AR transcriptional activity induced by DHT.

Group <sup>a</sup> , compound	RIC <sub>20</sub> <sup>b</sup> (M)	RLA <sup>c</sup> (%)	Group <sup>a</sup> , compound	RIC <sub>20</sub> <sup>b</sup> (M)	RLA <sup>c</sup> (%)
DHT alone		100	Fenthion	$4.9 \times 10^{-6}$	46
1. <i>o,p'</i> -DDT	$5.5 \times 10^{-7}$	7	Cyanophos	$5.5 \times 10^{-6}$	63
<i>p,p'</i> -DDE	$6.5 \times 10^{-7}$	6	Leptophos	$5.7 \times 10^{-6}$	53
<i>p,p'</i> -DDT	$7.1 \times 10^{-7}$	15	Bromophos-ethyl	$7.4 \times 10^{-6}$	68
Chloropropylate	$7.2 \times 10^{-7}$	7	Quinalphos	$7.8 \times 10^{-6}$	71
Chlorobenzilate	$1.2 \times 10^{-6}$	16	Isofenphos	$8.7 \times 10^{-6}$	75
Heptachlor epoxide	$1.3 \times 10^{-6}$	63	MEP oxon	$9.4 \times 10^{-6}$	78
Dicofol	$1.6 \times 10^{-6}$	20	4. Flucythrinate	$6.6 \times 10^{-6}$	68
<i>p,p'</i> -DDD	$1.8 \times 10^{-6}$	22	Fenvalerate	$6.9 \times 10^{-6}$	64
$\beta$ -Endosulfan	$2.0 \times 10^{-6}$	61	Cyfluthrin	$8.4 \times 10^{-6}$	74
Methoxychlor	$2.1 \times 10^{-6}$	18	Etofenprox	$9.2 \times 10^{-6}$	77
<i>trans</i> -Chlordane	$2.4 \times 10^{-6}$	63	5. Methiocarb	$2.8 \times 10^{-6}$	61
<i>cis</i> -Chlordane	$2.5 \times 10^{-6}$	42	Thiobencarb	$9.4 \times 10^{-6}$	78
Dieldrin	$2.8 \times 10^{-6}$	53	6. Thienylchlor	$5.4 \times 10^{-6}$	64
$\alpha$ -Endosulfan	$6.9 \times 10^{-6}$	66	Mefenacet	$5.6 \times 10^{-6}$	65
2. Chlornitrofen	$4.3 \times 10^{-8}$	19	Alachlor	$9.6 \times 10^{-6}$	79
Chlomethoxyfen	$6.8 \times 10^{-8}$	1	7. Propanil	$1.4 \times 10^{-6}$	27
Nitrofen	$3.4 \times 10^{-7}$	4	Pencycuron	$1.5 \times 10^{-6}$	19
CNP-amino	$8.3 \times 10^{-7}$	12	Linuron	$2.0 \times 10^{-6}$	37
Oxyfluorfen	$8.7 \times 10^{-7}$	8	Prochloraz	$3.4 \times 10^{-6}$	43
Bifenox	$3.2 \times 10^{-6}$	43	Diuron	$8.7 \times 10^{-6}$	75
Acifluorfen-methyl	$8.9 \times 10^{-6}$	76	9. Vinclozolin	$1.6 \times 10^{-7}$	0.3
3. Fenitrothion	$1.8 \times 10^{-7}$	2	Procymidone	$2.0 \times 10^{-7}$	1
Anilofos	$1.9 \times 10^{-6}$	13	Bromopropylate	$5.3 \times 10^{-7}$	4
EPN	$1.9 \times 10^{-6}$	35	Pendimethalin	$1.2 \times 10^{-6}$	10
Prothiofos	$2.2 \times 10^{-6}$	23	Bitertanol	$2.6 \times 10^{-6}$	45
Parathion	$2.2 \times 10^{-6}$	31	Triflumizole	$3.5 \times 10^{-6}$	37
Methyl parathion	$2.3 \times 10^{-6}$	29	Imazalil	$4.2 \times 10^{-6}$	51
Tolclofos-methyl	$2.8 \times 10^{-6}$	49	2-Phenylphenol	$4.9 \times 10^{-6}$	59
Piperophos	$3.0 \times 10^{-6}$	38	Pyrazoxifen	$5.0 \times 10^{-6}$	59
Ethion	$3.3 \times 10^{-6}$	28	Propiconazole	$6.2 \times 10^{-6}$	60
Butamifos	$3.3 \times 10^{-6}$	46	Fenarimol	$7.0 \times 10^{-6}$	65
Phosalone	$4.5 \times 10^{-6}$	46	Ethoxyquin	$7.8 \times 10^{-6}$	71
Dichlofenthion	$4.8 \times 10^{-6}$	57			

Abbreviations: EPN, *O*-ethyl *O*-*p*-nitrophenyl phenylphosphonothioate; RLA, relative luciferase activity.

<sup>a</sup>Nine compound groups are listed in Table 1. <sup>b</sup>Concentration of the test compound showing 20% inhibition of the androgenic activity induced by  $10^{-10}$  M DHT. <sup>c</sup>Percentage response at a concentration of  $10^{-5}$  M with 100% activity defined as the activity achieved with  $10^{-10}$  M DHT.

**Table 5.** Thirty-four pesticides possessing both estrogenic and antiandrogenic activities *in vitro*.

Pesticide	hER $\alpha$	hER $\beta$	hAR
Group 1			
<i>cis</i> -Chlordane	↑	↑	↓
<i>trans</i> -Chlordane	↑↑	↑↑	↓
<i>o,p'</i> -DDT	↑↑	↑↑	↓↓
<i>p,p'</i> -DDT	↑	↑	↓↓
<i>p,p'</i> -DDE	↑	↑	↓↓
<i>p,p'</i> -DDD	↑	↑	↓
Chlorobenzilate	↑	—	↓
Chloropropylate	↑	↑	↓↓
Dicofol	↑	↑	↓
Dieldrin	↑↑	—	↓
$\alpha$ -Endosulfan	↑↑	↑	↓
$\beta$ -Endosulfan	—	↑	↓
Heptachlor epoxide	↑	↑	↓
Methoxychlor	↑↑	↓	↓
Group 2			
Chlornitrofen (CNP)	↑	—	↓↓
CNP-amino	↑↑	↑↑	↓↓
Group 3			
Bromophos-ethyl	↑↑	↑	↓
Butamifos	↑↑	—	↓
Dichlofenthion	↑	↑	↓
EPN	↑	↑	↓
Ethion	↑	↑	↓
Isofenphos	↑	—	↓
Leptophos	↑	↑	↓
Prothiofos	↑	↑	↓
Quinalphos	↑	↑	↓
Tolclofos-methyl	↑	↑	↓
Group 4			
Fenvalerate	↑	—	↓
Cyfluthrin	↑	—	↓
Flucythrinate	↑	—	↓
Group 5			
Methiocarb	↑	↑↑	↓
Group 6			
Thienylchlor	↑	↑	↓
Group 9			
Bromopropylate	↑	↑	↓↓
Fenarimol	↑	↑	↓
Pendimethalin	↑	↑	↓

Symbols: ↑↑, agonistic effect ( $\text{REC}_{20} \leq 10^{-6}$  M); ↑, agonistic effect ( $10^{-6}$  M <  $\text{REC}_{20} \leq 10^{-5}$  M); ↓↓, antagonistic effect ( $\text{RIC}_{20} \leq 10^{-6}$  M); ↓, antagonistic effect ( $10^{-6}$  M <  $\text{RIC}_{20} \leq 10^{-5}$  M); —, no effect. EPN, *O*-ethyl *O*-*p*-nitrophenyl phenylphosphonothioate.

oxamyl) decreased estrogen- or progesterone-responsive reporter genes at concentrations of  $10^{-7}$  M in breast (MCF-7) and endometrial (Ishikawa) cancer cells. However, Andersen et al. (2002) reported that methomyl induced no significant effects in proliferation and ER transactivation assays using MCF-7 cells and that methiocarb showed both estrogenic and weak antiandrogenic properties. In our study, of the 22 carbamates tested only methiocarb (see Figure 4) showed both estrogenic and antiandrogenic activities, and other carbamate pesticides showed no ER or AR activity. Thus, our results support the evidence of Andersen et al. (2002) but not that of Klotz et al. (1997).

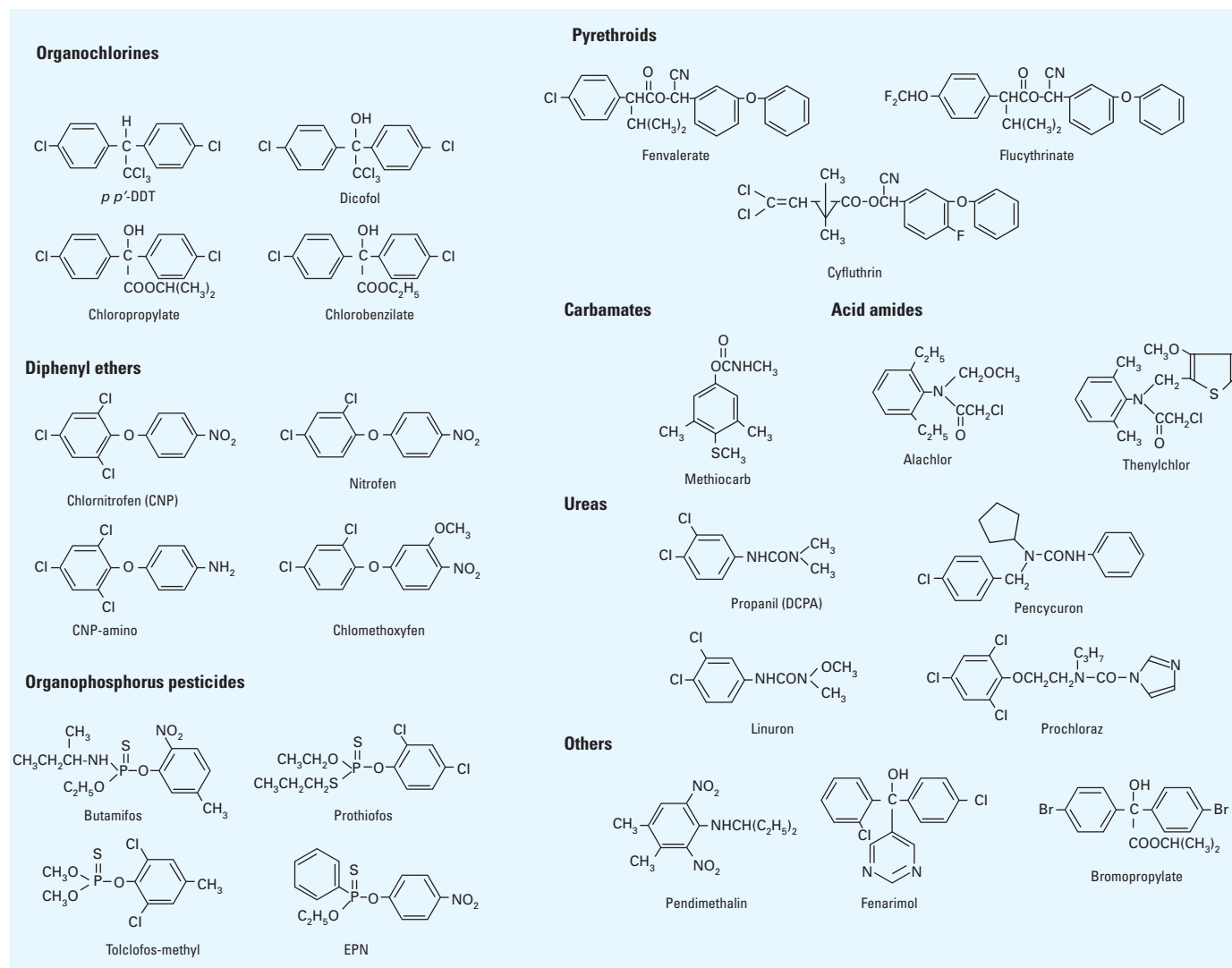
With regard to acid amide-type pesticides, only one relevant study was available. Vonier et al. (1996) reported the interaction of the herbicide alachlor with the estrogen and progesterone receptors from the oviduct of the American alligator. They showed that this pesticide competed with  $E_2$  for binding to the ER, but the binding affinity was about

3,500-fold lower than that of  $E_2$ . In our assays using hERs and hAR, alachlor showed both antiestrogenic activity via ER $\alpha$  and antiandrogenic activity. This suggests that alachlor can interact not only with alligator ER but also with hER $\alpha$  and hAR. Furthermore, among 13 acid amide pesticides, we newly found thenylchlor to have both estrogenic and antiandrogenic activities, and mefenacet to possess antiandrogenic activity.

Urea-type pesticides are mainly used as herbicides. Bauer et al. (1998) reported evidence that propanil (DCPA), linuron, and diuron in phenyl urea herbicide have the ability to bind to AR. In addition, recent reports have shown linuron and prochloraz to be antiandrogenic *in vitro* and *in vivo* (Lambright et al. 2000; Vinggaard et al. 2002). In our AR assay, five urea-type herbicides (DCPA, pencycuron, linuron, prochloraz, and diuron) inhibited transcriptional activity by DHT, and the antiandrogenic activities of DCPA and pencycuron were more potent than those of linuron

and prochloraz, which have been shown to be antiandrogenic *in vivo*. On chemical structure, these pesticides have some similarities (Figure 4). These suggest that DCPA and pencycuron would also show antiandrogenic activity *in vivo* and should therefore be considered endocrine disruptors.

Among the triazine-type pesticides, atrazine, which is the most widely used herbicide in the United States, has been reported to be antiestrogenic by yeast transactivation assay (Tran et al. 1996). In the present study, seven triazine-type pesticides were tested, but none showed any ER or AR activity. Friedmann (2002) recently reported that atrazine acts as an endocrine disruptor in rat males by directly inhibiting Leydig cell testosterone production. Hayes et al. (2002) hypothesize that atrazine induces aromatase and promotes the conversion of testosterone to estrogen in *Xenopus laevis*. Thus, this type of pesticide may exert hormonal activity through mechanisms other than those associated with ER and AR.



**Figure 4.** Chemical structures of some exemplar pesticides showing estrogenic and/or antiandrogenic activity.

In the present study, 44 pesticides that could not be classified into the above eight groups were collectively grouped as "others." The dicarboximide fungicides vinclozolin and procymidone are structurally similar and showed potent antiandrogen effects. This result corresponded to those of other studies (Kelce et al. 1994; Ostby et al. 1999). In addition, bromopropylate, fenarimol, and pendimethalin showed both estrogenic and antiandrogenic properties (Figure 4). Because the chemical structures of bromopropylate and chloropropylate are similar to those of DDT isomers, it was thought that the two pesticides would have similar effects. The *in vitro* estrogenic and antiandrogenic effects of the fungicide fenarimol have been also described by Andersen et al. (2002). The effects of fenarimol in our assays were more potent than described in that study, likely because of the difference in sensitivity of the assay systems. The herbicide pendimethalin has not been reported as having endocrine-disrupting effects, and thus we are the first to demonstrate the effects of this pesticide.

To date, no AR agonists have been found among environmental chemicals, and in this study we also failed to isolate an AR agonist from among 200 pesticides tested but identified 66 antiandrogenic pesticides. In addition, although there are many ER agonistic pesticides, there are also quite a few pesticides with antiestrogenic properties. This phenomenon is, as Sohoni and Sumpter (1998) pointed out, quite enigmatic. Furthermore, we demonstrated that a lot of pesticides possessed both estrogenic and antiandrogenic activities. Taken together, most of these chemical compounds may act as ER agonists and/or AR antagonists in the environment, a situation leading to feminization in animals.

Our experiments demonstrate that many pesticides possess *in vitro* estrogenic and antiandrogenic activities through ERs and/or AR. Although it appears that various pesticides exert hormonal effects at concentration-orders of magnitude higher than that required for physiologic hormones, wide exposure to large numbers of these pesticides may have additive and synergistic effects.

The first aim of this study was the comprehensive evaluation of 200 pesticides for *in vitro* estrogenicity and androgenicity under the same conditions using one highly sensitive and specific assay method. If different cells and plasmids were used in the assay, different results may be produced. However, we believe that the reporter gene assays in the present study are useful for identifying endocrine disruptors via ERs and AR from a large number of chemicals. Such hormonal effects are expected to be found not only in pesticides but also in other chemicals in the environments. The second aim was the search for a relationship between chemical structure and

hormonal activity. In fact, we found it in a number of pesticides. This is an important point in identifying endocrine disruptors from the multitude of chemicals commonly in use. We herein propose that many compounds should be tested using the same method and under the same conditions to prevent confusion resulting from the use of different methods, and an international agreement should be reached for this purpose.

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